REMARKS

These remarks are in response to the Office Action mailed April 7, 2009. The Office Action indicates that claims 14-20 are withdrawn in the Office Action Summary. Applicants respectfully submit that claims 1-26 were previously pending in the application and claims 14-26 currently stand withdrawn.

Claims 2, 4, 6, 8 and 10 have been amended. Support for the amendments to the claims can be found throughout the specification as filed. The "NH2-" and "-COOH" have been removed from the claims to be consistent with the specification and remainder of the claims in view of the description that the sequence "contains" and "comprising". No new matter is believed to have been introduced.

I.   ELECTION/RESTRICTION

Applicants confirm the Election of Group I, claims 1-13, directed to a method of inhibiting the spread and/or reducing the risk of infection of a virus, classified in class 514, subclass 2. Applicants further confirm the election of the species of SEQ ID NO:13. Applicants acknowledge the Examiner's withdrawal of the species restriction and that the search was extended pursuant to Markush practice. Furthermore, Applicants acknowledge the Examiner's indication that the claims are free of the art.

II.   REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Claims 1-13 stand rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the enablement requirement. The claims allegedly contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Applicants respectfully traverse this rejection.

The Examiner has applied the "Wands" factors in considering the enablement of the presently claimed invention. Applicants respectfully provide the following remarks in a format consistent with the Office Action.

(1) **Nature of the invention** – The invention relates to cathelicidin functional fragments and their use as antiviral agents.

(2) **The State of the Prior Art** – The Office Action alleges that the art shows significant obstacle in achieving viral vaccination. In particular, the Office Action uses HIV as an example to demonstrate the lack of enablement of the presently claimed invention. In order to ease the Examiner's burden, Applicants will not reiterate the Examiner's review of the literature as set forth in the Office Action, but would refer to pages 7-8 for the Office's position that the ability to treat HIV is difficult and tremendously challenging.

Applicants respectfully submit that the present invention is useful in and enabled for the treatment of viral contamination and treatment. As an example, and particularly relevant to the rejection and analysis in the Office Action, Applicants direct the Examiner to Wang *et al.*, "Anti-Human Immunodeficiency Virus Type 1 Activities of Antimicrobial Peptides Derived from Human and Bovine Cathelicidins," Antimcirobial Agents and Chemotherapy, Sept. 2008, Vol. 52, No. 9, pp. 3438-3440 (attached hereto as Appendix A).

Wang *et al.* is not prior art to Applicants' invention. Wang *et al.* demonstrates that "functional fragments of cathelicidin" are effective therapeutics against HIV. Wang *et al.* demonstrate the function fragments including KR-20 containing the consensus sequence of SEQ ID NO:1 have activity against HIV.

In addition, Braff *et al.*, "Structure-Function Relationships among Human Cathelicidin Peptides: Dissociation of Antimicrobial Properties from Host Immunostimulatory Activities," J. of Immunol., 2005, pp. 4271-4278, Vol. 174 (attached hereto as Appendix B), demonstrates that fragments of cathelicidin are effective at killing vaccinia virus (see, *e.g.*, Table II).

Numerous other references demonstrate that cathelicidins have anti-viral activities, see, *e.g.*, Gordon *et al.*, Curr. Eye Res. 2005, pp. 385-394, Vol. 30, No. 5; Howell *et al.*, J. Allergy Clin. Immunol., pp. 836-841, Vol. 117, No. 4; Howell *et al.*, J. Immunol., 2004, pp. 1763-1767, Vol. 172; and Bergman *et al.*, Curr. HIV Res., 2007, pp. 410-415, Vol. 5, No. 4. Accordingly, Applicants submit that the conceived invention as-claimed is enabled as demonstrated not only by Applicants' disclosure

but also by after filing references demonstrating the effectiveness of the claimed methods and compositions for treating, for example, HIV, HSV, and vaccinia viruses.

(3) **The relative skill of those in the art** – The skill in the art is high. Accordingly, methods of screening, synthesizing and modifying (*e.g.*, by addition of D- amino acids, for example), can be performed with ease. Peptide fragments can be easily synthesized and screened using methods described in the specification and in the art.

(4) **The predictability or unpredictability of the art** – Given the state of the art as set forth in Appendix A, B and the literature cited above, Applicants respectfully submit that the methods of use and the synthesis of the peptides can be readily performed.

(5) **The breadth of the claims** – Applicants respectfully submit that the breadth of the claims is enabled by reference to the specification, skill in the art, and the demonstration of the methods as described above.

(6) **The amount of direction or guidance presented and (7) the presence of absence of working examples** – Applicants respectfully submit that working examples are not required for enablement. Furthermore, Applicants have effectively described and demonstrated that peptides comprising the core sequence KRIVQRIKDFLRNLFP (as well as other peptide fragments of cathelicidin) are useful in the methods for treating, inhibiting or reducing the risk of viral infections.

(8) **The quantity of experimentation necessary** – As mentioned above, the skill in the art is high. The cathelicidin polypeptide is not extremely large and a number fragments have been demonstrated to work. Methods of screening additional fragments and modified peptide fragments (*e.g.*, those comprising unnatural or D- amino acids) can be readily performed using methods and techniques known to one of skill in the art with reference to the specification as-filed. Such experimentation is

not undue, but merely routine and easily performed based upon the teachings of the present application and techniques in the art.

For at least the foregoing, the Applicant submits that the claimed invention is patentable and request reconsideration and notice of such allowable subject matter. The Examiner is respectfully requested to call the undersigned following review of the present response so that prosecution may be advanced more readily and effectively.

The Director is authorized to charge any required fee or credit any overpayment to Deposit Account Number 50-4586, please reference the attorney docket number above.

Respectfully submitted,

GAVRILOVICH, DODD & LINDSEY LLP

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APPENDIX A

Anti-Human Immunodeficiency Virus Type 1 Activities of Antimicrobial Peptides Derived from Human and Bovine Cathelicidins[▽]

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From among 15 human cathelicidin LL-37-derived peptides, FK-13 was identified as the smallest peptide active against human immunodeficiency virus (HIV) and GI-20 had the highest therapeutic index, which was twice that of LL-37. BMAP-18, which is derived from bovine cathelicidin BMAP-27, possessed a therapeutic index similar to that of GI-20. Peptide sequence order, helical structures, and aromatic residues are important in HIV inhibition.

AIDS has become the fourth leading cause of death worldwide, and the majority of human immunodeficiency virus (HIV) infections are acquired through heterosexual intercourse. The United Nations estimates that there are now 40 million people living with HIV infection or AIDS. Thus, it is urgent that novel therapeutic and preventative agents, including topical microbicides that block the sexual transmission of HIV, be developed (5, 26). Antimicrobial peptides are ancient and potent host defense molecules in nearly all forms of life (3, 12, 37). More than 870 such peptides have been registered in the updated antimicrobial peptide database (http://aps.unmc.edu/AP/main.html) (33). However, only a few have been subjected to antiviral assays (8, 12). Known examples are brevinin-1 (35), caerin 1.1, caerin 1.9, and maculatin 1.1 (27), dermaseptin S4 (15), esculentin 2P and ranatuerin 2P (7), and the magainins (17) from amphibians and cecropin A and melittin from insects (28). In mammals, defensins and cathelicidins are the two major classes of antimicrobial peptides. While the three types of defensins, the α-, β-, and θ-defensins, contain several β strands, which are further stabilized by three pairs of disulfide bonds, the cathelicidins vary in both their sequences and their three-dimensional structures (usually extended or α-helical structures). Another important difference is that there are at least 10 different defensins in humans, but only one cathelicidin (LL-37) has been identified (29, 36). All human α-defensins and human β-defensin-3 inhibit HIV infection (11), but the θ-defensins are more effective (9, 10, 18, 31, 32). Cathelicidins have been shown to have effects on bacteria, fungi, and viruses (36). Among them, LL-37 (2), protegrin-1 (25), and indolicidin (16, 23) have been demonstrated to have anti-HIV activities. As the only cathelicidin in humans, LL-37 can be cleaved in vivo into active fragments. While KS-30, KR-20, and RK-31 were identified in human sweat, LL-23,

KS-27, and LL-29 were detected in human skin (19, 34). Furthermore, several laboratories have identified active fragments within LL-37 by synthesizing peptides corresponding to different regions (4, 20, 21, 29). Using nuclear magnetic resonance spectroscopy, we previously identified a minimally antimicrobial and anticancer region corresponding to residues 17 to 29 (FK-13 in Table 1) (13). Here, we report on the anti-HIV activities of 20 synthetic peptides (>95% purity; Genemed Synthesis, Inc.) derived from human and bovine cathelicidins.

Inhibition assays for determination of the anti-HIV cytopathic effect were conducted as described previously (6). Briefly, serially diluted peptides were added to a 96-well round-bottom microtiter plate in triplicate. CEM-SS cells at a concentration of 2.5×10^3 cells per well and HIV type 1_{IIIB} (HIV-1_{IIIB}) at the appropriate predetermined titer were sequentially added to the microtiter plate. The cultures were incubated at 5% CO_2 and 37°C for 6 days. Following the incubation, the microtiter plates were stained with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide dye to evaluate the efficacy and toxicity of the test compound(s). By using the Microsoft Excel program, the 50% effective concentration for inhibition of virus replication (EC_{50}), the concentration that reduced cell viability by 50% (TC_{50}), and a therapeutic index (TI; which is equal to TC_{50}/EC_{50}) were obtained. Consistent with the findings of Bergman et al. (2), we found that synthetic LL-37 is active against HIV-1_{IIIB} (Table 1). However, LL-23 and KR-20, the naturally occurring N- and C-terminal fragments of LL-37, respectively, showed no effect on the virus even at a high peptide concentration of 100 µg/ml (in µM in Table 1), suggesting that the HIV-active region of LL-37 is located in the middle region. Indeed, SK-21, which is composed of LL-37 with 8 residues truncated from each end, was active. We predict that naturally occurring LL-37 fragments, such as KS-27, LL-29, KS-30, and RK-31, inhibit HIV, as they all contain the sequence of SK-21. Inactive terminal fragments LL-23 and KR-20 were apparently not produced to protect humans from HIV or retrovirus infection, but they are bactericidal (19, 34).

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TABLE 1. Anti-HIV activities of human cathelicidin LL-37-derived peptides in CEM-SS cells[a]

Peptide name	Peptide sequence[b]	EC_{50} (μM)	TC_{50} (μM)	TI
LL-37	LLGDLLRKSKEK<u>IGKEFKRIVQRIKDFLRNL</u>VPRTES	1.6	18.4	11.5
LL-23	LLGDLLRKSKEKIGKEFKRIVQR	>35.4	>35.4	
KR-20	KRIVQRIKDFLRNLVPRTES	>40.5	>40.5	
SK-21	SKEKIGKEFKRIVQRIKDFLR	10.8	22.5	2.08
FK-13	FKRIVQRIKDFLR	3.4	10.4	3.1
Retro-FK-13	RLFDKIRQVIRKF	>58.1	33.7	
KR-12	KRIVQRIKDFLR	>63.5	>63.5	
GF-17	GFKRIVQRIKDFLRNLV	0.98	8.9	9.1
GF-17$_{d1}$[c]	GFKRIVQRIKDFLRNLV	>47.5	22.7	
GF-17$_{d2}$[c]	GFKRIVQRIKDFLRNLV	>47.5	>47.5	
GI-20	GIKEFKRIVQRIKDFLRNLV	1.08	22.7	21
GI-20$_{X17}$	GIKE**X**KRIVQRIKDFLRNLV	>40.6	7.3	
GI-20$_{W17}$	GIKE**W**KRIVQRIKDFLRNLV	7.4	23.6	3.2
GI-20$_{Q16}$	GIK**Q**FKRIVQRIKDFLRNLV	0.91	13.7	15.1
GI-20$_{EF}$	GIKEFKR**EF**QRIKDFLRNLV	1.6	9.9	6.2

[a] Although the standard errors from multiple antiviral assays are not provided, they were, on average, less than 10% of the respective mean EC_{50} or TC_{50}.
[b] In the sequence of LL-37, the region used to engineer an optimal anti-HIV peptide (GI-20) is underlined. For all sequences, mutated residues are in boldface, and X represents phenylglycine.
[c] The incorporation of D-amino acids is indicated by d followed by the number of D-amino acids (in boldface).

Our previously identified 13-residue antibacterial peptide FK-13 (13) also displayed an effect on HIV (Table 1). When F-17 (as numbered in LL-37; Table 1) was removed, the resultant KR-12 peptide lost its activity, indicating that F-17 is essential and that FK-13 represents the minimal anti-HIV region of human LL-37. Furthermore, reversal of the FK-13 sequence (retro-FK-13) led to a peptide that was inactive against HIV, although the peptide retained its antibacterial activity (14). Thus, there is no correlation between the antibacterial and the anti-HIV activities of these peptides. To obtain peptide templates with improved TIs, additional peptides were designed on the basis of the sequence of FK-13. GF-17 (i.e., FK-13 plus the NLV segment) had a TI slightly less than that of LL-37, whereas GI-20 (30), obtained by adding GIKE to the N terminus of GF-17, had a TI twice that of LL-37. We found that the incorporation of D-amino acids into GF-17 at position 24 (GF-17$_{d1}$) or both position 24 and position 28 (GF-17$_{d2}$) disrupted the activity of the peptide, but the cytotoxicity of the peptide to human cells decreased with an increase in the number of D-amino acids (Table 1). Since D-amino acid incorporation tends to reduce helicity (13, 22), the putative helical structure of GF-17 might be important for anti-HIV activity. In the case of GI-20, a change of F-17 to phenylglycine (GI-20$_{X17}$ in Table 1) led to the loss of activity. Likewise, a change of F-17 to W (GI-20$_{W17}$ in Table 1) also reduced the anti-HIV activity of the peptide. These results further substantiated the essential role of F-17 of LL-37 in inhibiting HIV infection. Interestingly, when E-16 of GI-20

was replaced by Q-16 (GI-20$_{Q16}$ in Table 1), the peptide became more toxic to human cells without any change in the anti-HIV activity observed. Thus, E-16 endowed selectivity to the peptide. Since F-17 is critical for the anti-HIV effect and E-16 modulates selectivity, we also tested the effects of this amino acid pair by substituting I19V20 for E19F20 (GI-20$_{EF}$ in Table 1). While the antiviral efficacy of the resulting peptide was reduced only slightly, cellular toxicity increased twofold relative to that of GI-20. Clearly, the effect of F overrode the effect of E in this case. Hence, both the anti-HIV efficacy and the cellular toxicity of GI-20 are subjected to modulation, laying the basis for peptide engineering.

To obtain additional peptide templates, we also evaluated the anti-HIV activity of a few peptides (Table 2) based on BMAP-27. BMAP-27 is a 27-residue bovine cathelicidin peptide with the potential to form an α-helical conformation followed by a hydrophobic tail (24). To identify the region within BMAP-27 active against HIV, we first deleted the hydrophobic tail to obtain BMAP-18. BMAP-18 was found to be more active against HIV than GI-20, but it was also more toxic to human cells than GI-20, although the overall TI of BMAP-18 was slightly better (Table 2). Further deletion of the three residues from the C terminus of BMAP-18 disrupted both the antiviral and the cytotoxic effects of the resulting peptide (BMAP-15). Subsequent mutational studies revealed that changing F-6 and F-10 of BMAP-18 to phenylglycines slightly reduced the anti-HIV activity of BMAP-18. The peptide became inactive when the two phenylalanines were changed to

TABLE 2. Anti-HIV activities of bovine cathelicidin BMAP-27-derived peptides in CEM-SS cells[a]

Peptide name	Peptide sequence[b]	EC_{50} (μM)	TC_{50} (μM)	TI
BMAP-18	GRFKRFRKKFKKLFKKIS	0.35	8.45	24.1
BMAP-18$_{P9}$	GRFKRFRK**P**FKKLFKKIS	3.20	18.9	5.9
BMAP-18$_{X6X10}$	GRFKR**X**RKK**X**KKLFKKIS	0.68	10.2	15
BMAP-18$_{16L10}$	GRFKR**I**RKK**L**KKLFKKIS	>44.0	2.79	
BMAP-15	GRFKRFRKKFKKLFK	>49.6	>49.6	

[a] Although the standard errors from multiple antiviral assays are not provided, they were, on average, less than 10% of the respective mean EC_{50} or TC_{50}.
[b] Mutated residues are in boldface, and X represents phenylglycine.

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isoleucine/leucine residues, suggesting that the aromatic rings of BMAP-18 are critical for its anti-HIV activity. Also, the antiviral effect was decreased when K-9 of BMAP-18 was replaced with a proline residue. The introduction of a proline (1, 10) could distort the helical structure of the peptide (24), which might also be critical for anti-HIV activity.

In conclusion, our evaluation of 20 synthetic peptides derived from LL-37 and BMAP-27 led to the identification of the most important regions in both human and bovine cathelicidins active against HIV. Peptide sequence order, aromatic phenylalanine residues, and potential helical structures were found to play important roles in blocking HIV-1 infection. Because GI-20 and BMAP-18 have TIs superior to the TI of LL-37, they may now be used as templates for the engineering of novel anti-HIV microbicides.

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APPENDIX B

Structure-Function Relationships among Human Cathelicidin Peptides: Dissociation of Antimicrobial Properties from Host Immunostimulatory Activities

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Cathelicidins and other antimicrobial peptides are deployed at epithelial surfaces to defend against infection. These molecules have broad-spectrum killing activity against microbes and can have effects on specific mammalian cell types, potentially stimulating additional immune defense through direct chemotactic activity or induction of cytokine release. In humans, the cathelicidin hCAP18/LL-37 is processed to LL-37 in neutrophils, but on skin it can be further proteolytically processed to shorter forms. The influence of these cathelicidin peptides on keratinocyte function is not known. In the current study, DNA microarray analysis and confirmatory protein analysis showed that LL-37 affects the expression of several chemokines and cytokines by keratinocytes. Analysis of a synthetic peptide library derived from LL-37 showed that antimicrobial activity against bacterial, fungal, and viral skin pathogens resides within specific domains of the parent peptide, but antimicrobial activity does not directly correlate with the ability to stimulate IL-8 production in keratinocytes. IL-8 release was induced by D- and L-amino acid forms of cathelicidin and correlated with membrane permeability, suggesting that highly structure-specific binding to a cell surface receptor is not likely. However, this effect was inhibited by either pertussis toxin or AG1478, an epidermal growth factor receptor tyrosine kinase inhibitor, suggesting that cathelicidin may indirectly stimulate multiple signaling pathways associated with cell surface receptors. Taken together, these observations suggest that proteolytic processing may alter the balance between cathelicidin antimicrobial and host immunostimulatory functions. *The Journal of Immunology*, 2005, 174: 4271–4278.

E pithelial interfaces with the external environment rely on innate immune defense mechanisms for rapid recognition and defense against infection. The skin is an important example, acting as a physical and chemical barrier against external pathogens in a variety of extreme situations such as wound repair, where infection becomes a major cause of morbidity and mortality. Individual cells comprising the skin are actively involved in this process (1). One way these individual cells are involved is through production and release of antimicrobial peptides (2–4).

In the skin, antimicrobial peptides can be released by resident cells such as keratinocytes, mast cells, and eccrine gland epithelia or are deposited by recruited cells such as neutrophils (5). Cathelicidins comprise one such family of antimicrobial peptides and exert their effects on a broad range of microbes, including Gram-positive and -negative bacteria as well as fungi and enveloped viruses (6, 7). Humans have a single cathelicidin gene encoding hCAP18/LL-37 (8, 9). In addition to being expressed by several cells of the skin, hCAP18/LL-37 is abundantly expressed in neutrophils and in multiple mucosal epithelia, such as airways (10), buccal mucosa, tongue, esophagus, cervix, vagina (11), and sali-

vary glands (12). In certain cell types, such as those found in eccrine sweat glands, cathelicidin is constitutively expressed (12). In other cells, such as keratinocytes, cathelicidin expression is inducible (3) and is rapidly up-regulated in the epidermis after injury (13). The role that cathelicidins play in host defense extends beyond their ability to directly kill pathogens. The hCAP18 precursor protein is processed upon neutrophil granule fusion to a 37-aa peptide form, LL-37, by cleavage with a serine protease (14). This form of the human cathelicidin has been shown to be involved in chemotaxis of mast cells, neutrophils, and CD4 T cells (15, 16) and can modify dendritic cell and keratinocyte function (17, 18). The chemotactic property of LL-37 has been associated with binding via formyl peptide receptor-like 1 (FPRL-1)[2] (19), which belongs to the G$_i$ protein-coupled receptor family. Other host cell activities appear to be independent of FPRL-1 and can involve upstream activation of P2X7 or epidermal growth factor receptors (EGFR) (20, 21).

Human cathelicidin peptides can exist naturally in forms other than LL-37. In sweat, three additional forms have been found: KR-20, a 20-aa derivative; RK-31, a 31-aa derivative; and KS-30, a 30-aa derivative. All three processed peptides exhibit an overall gain in antimicrobial activity compared with full-length LL-37 (22). The importance of processing human cathelicidin from LL-37 as the form first released from neutrophils to smaller peptide fragments is unclear.

To investigate the functional implications of LL-37 in the skin, the current study explored the gene expression profile of keratinocytes after exposure to LL-37 and found profound changes in cytokine and chemokine expression and release. The structural basis of this response was studied by generating a library of LL-37-derived synthetic peptide fragments. Observations from these experiments defined specific domains within LL-37 that are important for antibacterial, antifungal, and antiviral activity as well as host immunostimulatory activity. Peptides with the ability to stimulate keratinocytes did not completely correlate with those that were antimicrobial, suggesting that processing of cathelicidin peptides can be an important modifier of host cell immune responsiveness. Additional mechanistic studies suggested that membrane-active LL-37 peptides may indirectly stimulate keratinocyte cell surface receptors and activate downstream signaling cascades, leading to changes in gene expression by host cells.

Materials and Methods

Cell culture and virus source

Neonatal human epidermal keratinocytes (Cascade Biologics) were grown in EpiLife medium (Cascade Biologics) supplemented with 0.06 mM Ca^{2+}, 1% EpiLife defined growth supplement, and 1% penicillin/streptomycin (Invitrogen Life Technologies). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The Wyeth strain *Vaccinia* was obtained from the Centers of Disease Control and Prevention.

DNA microarray analysis

Normal human keratinocytes were cultured to confluence in 100-mm dishes and treated with 0 or 10 μM LL-37 for 8 h. Total RNA was extracted using the Qiagen RNeasy kit. The Veterans Administration Medical Center GeneChip Core Facility processed five HuGeneFL GeneChips (Affymetrix) using 250–300 ng of total RNA/chip. Two chips were used for control keratinocytes, and three chips were used for the 10 μM LL-37-treated keratinocytes. Data were analyzed using GeneChip operating software and GeneSpring (Silicon Genetics). Data were filtered by genes showing >3-fold change in peptide-treated vs medium alone, and by statistical significance with $p < 0.05$ by two-tailed Student's t test.

Cytokine expression analysis

Keratinocytes were cultured to preconfluence, confluence, or 2 days post-confluence and treated with 0, 3, or 10 μM LL-37 for 24 h. Cytokine release was measured using the LINCOplex Human Cytokine Multiplex kit according to the manufacturer's instructions (Linco Research). Briefly, the filter plate was blocked for 10 min, followed by the addition of standards, samples, and beads. The plate was sealed, covered with aluminum foil, and incubated with shaking for 1 h. Wells were then washed twice, and detection Ab was added. The plate was again sealed and covered with aluminum foil, then incubated with shaking for 30 min. Streptavidin-PE was added to each well, and the plate was incubated an additional 30 min. Wells were washed twice, and sheath fluid was added. The plate was shaken for 5 min, then read using Luminex[100].

Measurement of IL-1α release from keratinocytes

Normal human epidermal keratinocytes were cultured to confluence and treated with 0 or 10 μM LL-37 in keratinocyte medium for 1 or 24 h at 37°C. IL-1α production was determined by IL-1α ELISA (R&D Systems) according to the manufacturer's instructions. Briefly, 96-well plates were coated overnight with capture Ab. Wells were blocked for 1 h, after which standards and samples (1/10 dilution) were added, and the plate was incubated for 2 h. Wells were incubated with detection Ab for an additional 2 h, followed by streptavidin-HRP for 20 min in the dark. The plate was incubated with substrate solution for 20 min in the dark. Without washing, stop solution was added to each well, and the plate was immediately read at 450 nm with correction at 570 nm.

LL-37 and LL-37 peptide fragment synthesis

LL-37, LL-25, LL-20, LL-15, LL-12, GD-23, RK-19, EK-20, KR-20, VQ-17, EK-15, GK-17, EF-15, RK-31, and KS-30 peptides were commercially prepared by Synpep. These sequences were selected based on predicted α-helical domains of LL-37 from alignments of α-helical cathelicidin family members from various species. These peptides represent N- and C-

terminal LL-37 peptide fragments as well as those central domains conserved among species and include sequences from both α-helical domains and disrupted helices. Peptide amino acid sequences are given in Fig. 2. All synthetic peptides were purified by HPLC to >95% purity, and identity was confirmed by mass spectrometry. Peptides were tested for LPS contamination by *Limulus* assay according to the manufacturer's instructions.

Determination of minimal inhibitory concentration

Bacteria were grown overnight in sterile tryptic soy broth. Bacteria in log phase were suspended to 1×10^6 CFU/ml in 10% tryptic soy broth (30 g/l stock; Sigma-Aldrich) in 10 mM phosphate buffer (27.6 g/l NaH_2PO_4-H_2O and 53.65 g/l Na_2HPO_4-7H_2O, pH 7.4). Peptide was added and incubated overnight at 37°C. The OD was then measured at 600 nm. Peptide activity against *Candida albicans* was determined in Dixon medium (0.6% peptone, 4% malt extract, 1% glucose, 0.1% ox bile, and 1% Tween 80) in sterile 96-well plates (Corning Glass) at a final volume of 50 μl. The assay mixtures contained 1–2.5 × 10⁴ CFU/ml *C. albicans*, 20% Dixon medium, 0.6 mM phosphate buffer (pH 7.0), and 16 μg/ml chloramphenicol. Plates were incubated at 25°C for 24 h with peptides, and absorbance was read at 600 nm.

Antiviral assay

BS-C-1 (American Type Culture Collection; CCL-26) African green monkey kidney cells (2 × 10⁵ cells/well) were seeded in 24-well tissue culture plates in MEM-10% FCS and penicillin/streptomycin and allowed to grow overnight before the supernatant was removed and replaced with MEM-2.5% FCS for virus incubation. Peptides were diluted to the proper concentrations in 0.01× tryptic soy broth containing 10 mM sodium phosphate buffer (pH 7.4). Virus diluted in the same buffer was added to the peptides and incubated for 24 h at 37°C. Twenty microliters of the peptide/virus mixture was added to the cells in 0.5 ml of MEM-2.5% FCS and allowed to infect for 48 h for plaque development. The medium was removed, and 0.5 ml of 0.1% crystal violet in PBS was added to the wells for 5 min at room temperature. Wells were then aspirated and air-dried for visualization of plaques.

Measurement of IL-8 release from keratinocytes

Keratinocytes were cultured to confluence and treated with the peptide of interest in keratinocyte medium at 37°C. For the cathelicidin peptide fragment panel and dose-response assays, peptides were incubated with keratinocytes for 6 h. For the time-course assay, cathelicidin peptides were used at a concentration of 3 μM. Supernatants were removed and placed in a sterile 96-well plate for ELISA. IL-8 production was determined by ELISA (BD Biosciences) according to the manufacturer's instructions. Briefly, 96-well plates were coated with capture Ab and incubated overnight at 4°C. Wells were washed three times, blocked for 1 h at room temperature, and again washed three times. Standards and 1/20 dilutions of samples were added to each well and incubated for 2 h at room temperature, followed by five washes. Detection Ab was added and incubated for 1 h at room temperature. Wells were washed seven times. Substrate solution was added to each well, and plate was incubated for 30 min at room temperature in the dark. Stop solution was added to each well, and the OD was observed at 450 nm with correction at 570 nm. IL-8 secretion from keratinocytes treated with pertussis toxin (Sigma-Aldrich), the FPRL-1 agonist WKYMVM-NH₂ (Phoenix Pharmaceuticals), or the EGFR inhibitor AG1478 (Calbiochem) was assessed by IL-8 ELISA as described.

Quantitative real-time PCR

Keratinocytes were cultured to confluence and treated with the peptide of interest in keratinocyte medium for 6 h at 37°C. Total RNA was isolated from treated cells using an RNeasy Mini kit (Qiagen). Isolated RNA was retrotranscribed by reverse transcriptase using the Retroscript kit (Ambion). Briefly, first-strand synthesis combined RNA with 0.4 mM dNTPs and 4 μM random decamers in 16 μl at 80°C for 3 min. The reaction was iced, and 2 μl of 10× RT-PCR buffer, 1 μl of RNase inhibitor, and 1 μl of Moloney murine leukemia virus RT were added and incubated at 42°C for 1 h, then denatured at 92°C for 10 min. Real-time quantitative PCR was performed using an Applied Biosystems 7000 Sequence Detection System. TaqMan predeveloped assay reagents were used to detect human IL-8 (Applied Biosystems), and GAPDH primers and probe were used as an endogenous control. Briefly, 2 μl of the RT reaction was added to 25 μl of TaqMan Universal PCR Master Mix (Roche), 2.5 μl of target primers and probe, and 20.5 μl of RNase/DNase-free water. The thermal profile was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15 s, followed by 60°C for 1 min. Results were analyzed using the comparative cycle threshold method (Applied Biosystems). Briefly, cycle threshold values

were normalized to GAPDH, which was used as an endogenous control, then normalized to the sample chosen as a calibrator. Product specificity was confirmed by generating dissociation curves for the reactions.

Measurement of cell viability

Keratinocytes were incubated for 6 h at 37°C with 0, 3, or 10 μM cathelicidin peptide. Supernatants were collected into clean 96-well plates. Cells were washed three times. Basal medium (100 μl) was added to each well. Viability was assessed using the CellTiter96 Non-Radioactive Cell Proliferation Assay (MTT; Promega) according to the manufacturer's instructions. Briefly, dye solution (15 μl) was added to each well, and the plate was incubated 4 h at 37°C. Solubilization/stop solution (100 μl) was added to each well, and the plate was incubated in a dark, moist chamber overnight at room temperature. OD was determined at 570 nm, with correction at 660 nm. Alternatively, cells were incubated briefly with 1.3 μg/ml propidium iodide (PI) after removal of supernatants, and fluorescence intensity was measured using a fluorescence microplate reader at an excitation of 530 nm and an emission of 620 nm. For evaluation of toxicity by membrane permeability to lactate dehydrogenase (LDH), LDH release from cells was measured using the Cytotoxicity Detection kit (Roche). Briefly, 50 μl of catalyst/dye solution was added to 50 μl of cultured supernatant removed from the cells used for the MTT or PI assays described above. Plates were incubated for 25 min at room temperature. OD was determined at 492 nm, with correction at 620 nm.

Results

LL-37 induces cytokine and chemokine production by normal human keratinocytes

The human cathelicidin antimicrobial peptide LL-37 has been implicated in a variety of effects on the host immune system, including chemotaxis (16, 19), dendritic cell differentiation (17), and cytokine release (22). Epithelial surfaces such as the skin are exposed to abundant levels of LL-37 during the response to injury or infection. It was of interest to evaluate the response of epidermal keratinocytes to LL-37 at concentrations similar to those found in vivo (13). DNA microarray analysis of cultured keratinocytes exposed to LL-37 for 8 h revealed effects on the transcripts of various genes (Table I). Many genes involved in

inflammation, including those encoding IL-8, cyclooxygenase 2, pro-IL-1β, TNF-α-induced protein 3, and IL-6, were significantly up-regulated.

To confirm the microarray analysis data showing that keratinocytes stimulated with LL-37 up-regulate the expression of cytokines and chemokines, quantitative immunodetection assays were performed on culture supernatants from treated cells. Consistent with the results of the microarray, IL-6 and IL-8 protein levels increased. The production of TNF-α and GM-CSF also increased. No changes were seen in the abundance of IL-1β, IL-2, IL-4, or IFN-γ (Fig. 1A). For analysis of IL-1α, ELISA of cultured supernatant from LL-37-treated keratinocytes was performed and demonstrated a large increase in IL-1α release compared with cells treated with medium only (Fig. 1B). Assays were performed on preconfluent, confluent, and postconfluent cells in the presence of low or high calcium, and no significant differences were found (data not shown).

Derivatives of LL-37 have diverse antimicrobial activity

To compare the mechanisms used in antimicrobial activity to those responsible for activating keratinocytes, a library of LL-37-derived peptides was synthesized. The sequences of these peptides are shown in Fig. 2. Select peptides exhibited a broad range of antimicrobial activities against test organisms, which included Gram-negative and -positive bacteria, Vaccinia virus, and C. albicans (Table II). The peptide fragments RK-31 and KS-30 showed the highest overall antimicrobial properties, with minimal inhibitory concentrations ranging from 2 to 16 μM. Peptide fragment GD-23 exhibited the lowest minimal inhibitory concentration against Staphylococcus aureus mprF at 2 μM. LL-25, EK-20, KR-20, GK-17, RK-31, and KS-30 also inhibited the growth of S. aureus mprF better than LL-37. Under these culture conditions, several fragments showed higher activity than LL-37 against Escherichia coli,

Table I. *Change in transcript expression in LL-37-treated human keratinocytes[a]*

Transcripts Up-Regulated	Fold Increase	Transcripts Down-Regulated	Fold Increase
IL-8	11.32	Thioredoxin interacting protein	9.14
A kinase (PRKA) anchor protein (gravin) 12	9.26	Estrogen receptor 1	7.35
PG endoperoxide synthase 2 (PTGS2, COX2)	9.18	Ataxia-telangiectasia locus protein	4.84
Cyclooxygenase 2 gene	6.75	18S rRNA gene	4.69
Chemokine (CXC motif) ligand 3 (CXCL3)	5.94	Profilaggrin gene	4.49
Pro-IL-1β (IL1β) gene	5.91	BRCA1	4.31
GOS2 gene	5.09	Primase (PRIM1)	3.17
Urokinase-type plasminogen receptor (UPAR, URKR)	5.08	BRCA2 gene	3.00
IL-13 receptor, α2	4.76		
Pim-1 oncogene	4.74		
Putative chemokine receptor	4.68		
Decay accelerating factor for complement	4.55		
Chemokine (CXC motif) ligand 2 (CXCL2)	4.54		
Melanoma growth stimulatory activity (MGSA) gene (CXCL1, GRO1)	4.40		
Solute carrier family 2 (facilitated glucose transporter)	4.25		
Thioredoxin reductase 1	4.11		
Human tumor Ag (L6)	3.99		
Baculoviral IAP repeat-containing 1	3.83		
Aldehyde dehydrogenase 1	3.60		
Dual specificity phosphatase 1	3.60		
TNF-α-induced protein 3	3.45		
IL-6	3.45		
Urokinase inhibitor (PAI2) gene	3.33		
B-cell translocation gene 1, antiproliferative	3.09		
Bone morphogenetic protein 2	3.06		

[a] Keratinocytes grown to confluence were treated with 0 or 10 μM LL-37 for 8 h. Total RNA was extracted and analyzed for transcript change by HuGeneFL GeneChips. Approximately 250–300 ng of RNA was used for each chip. Fold change represents an average of the difference among five GeneChips: two for the control compared with three for the 10 μM LL-37-treated cells. The data were analyzed by GeneChip Operating Software (Affymetrix) and GeneSpring (Silicon Genetics). Statistical analysis was performed by the paired Student *t* test. All values shown are significant to *p* < 0.05.
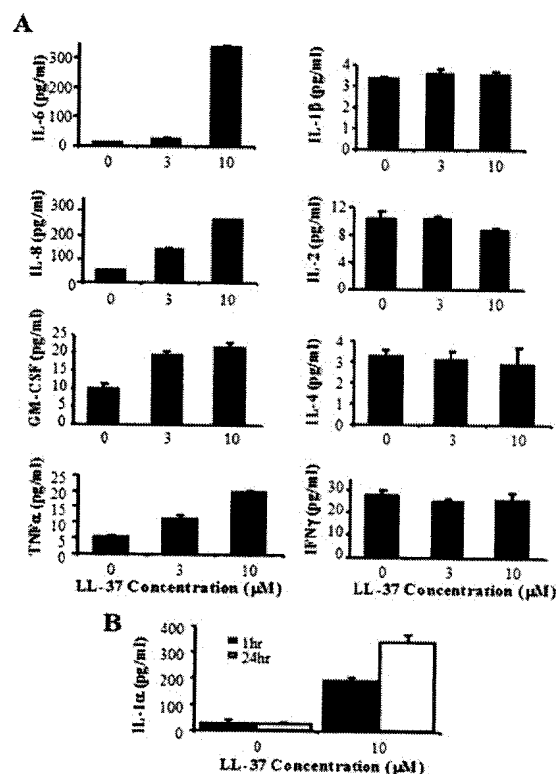
A

B

FIGURE 1. LL-37 influences cytokine production by human keratinocytes. LL-37 was diluted in keratinocyte medium to a final concentration of 3 or 10 μM. *A*, Keratinocytes were incubated with medium only or with LL-37 for 24 h at 37°C. Supernatants were analyzed for the indicated cytokines by Luminex assay. *B*, Keratinocytes were incubated with medium only or with 10 μM LL-37 for 1 or 24 h. Supernatants were analyzed for IL-1α by ELISA. Results are expressed as the mean ± SEM of one representative experiment of three, each performed in triplicate.

Name	Sequence
LL-37	LLGDFFRKSK EKIGKEFKRI VQRIKDFLRN LVPRTES
LL-25	LLGDFFRKSK EKIGKEFKRI VQRIK
LL-20	LLGDFFRKSK EKIGKEFKRI
LL-15	LLGDFFRKSK EKIGKE
LL-12	LLGDFFRKSK EK
GD-23	GDFFRKSK EKIGKEFKRI VQRIK
RK-19	RKSK EKIGKEFKRI VQRIK
EK-20	EKIGKEFKRI VQRIKDFLRN
KR-20	KRI VQRIKDFLRN LVPRTES
VQ-17	VQRIKDFLRN LVPRTES
EK-15	EKIGKEFKRI VQRIK
GK-17	GKEFKRI VQRIKDFLRN
EF-15	EFKRI VQRIKDFLRN
RK-31	RKSK EKIGKEFKRI VQRIKDFLRN LVPRTES
KS-30	KSK EKIGKEFKRI VQRIKDFLRN LVPRTES

FIGURE 2. Sequences of LL-37 and synthetic peptide fragments of LL-37. Peptides were commercially prepared, endotoxin free by *Limulus* assay, and purified by HPLC. Identity was confirmed by mass spectrometry.

including LL-25, GD-23, RK-19, EK-20, EK-15, GK-17, EF-15, RK-31, and KS-30.

Ability of cathelicidin peptides to stimulate IL-8 production correlates with reduction in keratinocyte viability, but is independent of peptide antimicrobial activity

The effects of LL-37 synthetic peptide fragments as well as the structurally divergent D-amino acid form of LL-37 were next compared with full-length LL-37 in assays for keratinocyte IL-8 production. For initial screening, keratinocytes were cultured for 6 h in the presence of peptides at a final concentration of 3 μM. D-LL-37 stimulated the highest production of IL-8 by keratinocytes, followed by LL-37, KS-30, and RK-31, respectively (Fig. 3).

Because EK-20, RK-31, and KS-30 were found to have good broad-spectrum antimicrobial activity, but little or no apparent ability to stimulate keratinocyte IL-8 release, dose and time responses were evaluated (Fig. 4). Treatment of keratinocytes with LL-37 resulted in the highest dose-dependent release of IL-8 from keratinocytes, although KS-30 also induced an increase in keratinocyte IL-8 production at higher concentrations. Peptide fragments RK-31 and EK-20 did not stimulate IL-8 above the medium background (Fig. 4*A*). To observe the time-dependent release of IL-8 by keratinocytes, peptides were diluted to 3 μM. IL-8 accumulated with time in both control and peptide-treated keratinocytes. Full-length LL-37 again stimulated the highest levels of IL-8 release from keratinocytes, demonstrating a 6-fold increase at 24 h

at a dose near the minimal IL-8 stimulatory concentration. EK-20, RK-31, and KS-30 displayed minimal effects on keratinocyte IL-8 production at the time points examined (Fig. 4*B*). The increasing accumulation of IL-8 over time in response to these peptides was similar to constitutive release from keratinocytes treated with medium alone.

To evaluate the effects of LL-37, EK-20, RK-31, and KS-30 on the membrane permeability of keratinocytes, LDH release and reduction of tetrazolium to formazan were studied by LDH and MTT assays, respectively. Minimal membrane disruption was observed by LDH assay for all peptides and concentrations tested (Fig. 5*A*). Keratinocyte viability, as evaluated by MTT assay, was clearly reduced upon exposure to 10 μM LL-37 and, to a lesser extent, RK-31 and KS-30 (Fig. 5*B*).

Stimulation of IL-8 production by LL-37 and D-LL-37 directly correlates with membrane permeability

Because 3 μM D-LL-37 was shown to exhibit potent IL-8 immunostimulatory ability in Fig. 3, dose-response experiments were performed to compare the activities of D-LL-37 and LL-37 (Fig. 6). Both LL-37 and D-LL-37, but not EK-20, increased IL-8 protein production in a dose-dependent manner (Fig. 6*A*). Maximal IL-8 induction occurred at a lower concentration of D-LL-37 (4 μM), but both peptides showed equivalent immunostimulatory activity at the highest dose of 10 μM. Therefore, although D-LL-37 appears to be more potent than LL-37, both peptides have equivalent maximal IL-8 stimulatory capacity. Quantitative real-time PCR analysis showed induction of IL-8 transcript by keratinocytes in response to LL-37 and D-LL-37, but not EK-20 (Fig. 7). It was interesting to note that IL-8 production in response to LL-37 and D-LL-37 correlated directly with membrane permeability, as measured by PI incorporation, suggesting that these events may be connected (Fig. 6*B*).

Cathelicidin peptide-induced IL-8 production in keratinocytes involves EGFR and Gᵢ protein signaling

The strict sequence requirement for keratinocyte immunostimulatory activity contrasted with the less stringent domain requirements for broad-spectrum antimicrobial activity. However, the potent effects of LL-37 synthesized with D-amino acids suggested that a traditional ligand-receptor binding mechanism for keratinocyte recognition of LL-37 was unlikely. Previous investigations have implicated FPRL-1, a Gᵢ-coupled receptor, in the chemotaxis of neutrophils, monocytes, and T cells in response to LL-37 (19). However, under the culture conditions used in this study, keratinocytes were not responsive to the FPRL-1 ligand WKYMVM-NH₂ at concentrations up to 100 times greater than those reported to be

Table II. *MIC (μM) of LL-37 and LL-37 synthetic peptide fragments[a]*

	MIC (μM)					
	E. coli (O29)	GAS (NZ131)	S. aureus (ATCC 25932)	S. aureus mprF	Vaccinia (Wyeth strain)	C. albicans (ATCC 14053)
LL-37	64	8–16	>64	32	32	16
LL-25	16	8	>64	8	>64	16
LL-20	>64	>32	>64	>32	>64	>32
LL-15	>64	>32	>64	>32	>64	>32
LL-12	>64	>32	>64	>32	>64	>32
GD-23	16	>32	>64	2	>64	32
RK-19	32	>32	>64	>32	>64	16
EK-20	32	8	>64	8–16	64	32
KR-20	>64	8	>64	8	>64	16
VQ-17	>64	>32	>64	>32	>64	>32
EK-15	>32	>32	>320	>32	>64	>32
GK-17	32	16	>320	16	32	16
EF-15	>32	32	>320	32	>64	32
RK-31	8	4	16	8	16	4
KS-30	8	4	16	8	8	2

[a] Bacterial and fungal minimal inhibitory concentration (MIC) values were determined by solution killing assay. Peptides ranging in concentration from 2 to 320 μM were added to bacteria in tryptic soy broth or to *C. albicans* in Dixon medium. Results indicate minimum concentration required for killing. Viral MIC was determined by the plaque method. Each assay was performed three times in triplicate, with representative data shown.

effective in other cell types (100 nM; data not shown). Similarly, FPRL-1 expression was not seen in cultured keratinocytes or in whole skin by immunostaining. This result agrees with recent studies demonstrating that FPRL-1 is not involved in LL-37-induced activation of epithelial cells (21) or monocytes (23).

Previous investigations showed that cell activation and signaling by LL-37 can be G_i protein dependent (19) or independent (23). Therefore, the IL-8 response was evaluated after treatment of keratinocytes with the G_i inhibitor, pertussis toxin (PTX), in the presence or the absence of LL-37 or D-LL-37. Because LL-37 and D-LL-37 have different potencies, peptide concentrations that induce equivalent levels of IL-8, 10 μM LL-37 and 4 μM D-LL-37, were chosen for inhibitor experiments. PTX significantly inhibited IL-8 release by keratinocytes in response to either LL-37 or D-LL-37 (Fig. 8). Because LL-37 has also previously been shown to transactivate the EGFR (21), IL-8 production was also assessed in keratinocytes treated with equivalent stimulatory doses of LL-37 and D-LL-37 in the presence of the EGFR tyrosine kinase inhibitor, AG1478. Similar to PTX, treatment with AG1478 significantly inhibited IL-8 release from keratinocytes in response to either LL-37 or D-LL-37 (Fig. 8). Taken together, these results suggest that LL-37 and D-LL-37 activate keratinocyte production of IL-8 through pathways involving both G_i protein signaling and EGFR activation.

Discussion

Cathelicidins, such as LL-37, possess an inherent ability to kill Gram-negative and -positive bacteria, fungi, and viruses. LL-37
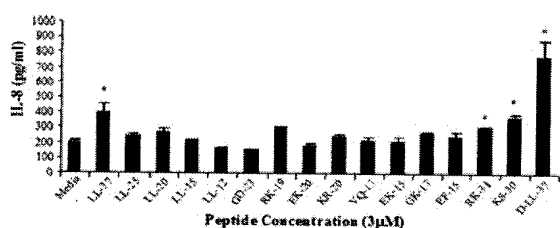
FIGURE 3. Specific LL-37 peptides influence IL-8 production by human keratinocytes. Peptides were diluted to a concentration of 3 μM in keratinocyte medium. Cells were incubated with peptide for 6 h at 37°C. Supernatants were removed and analyzed for IL-8 by ELISA. Results are expressed as the mean ± SEM of one representative experiment of three, each performed in triplicate. Statistical analysis was performed using the paired Student's t test: *, $p < 0.01$.

has also been shown to stimulate host inflammatory events (21, 24). The skin has demonstrated dependence on cathelicidin expression for resistance to infection by *Streptococcus pyogenes* in mice (25), and cathelicidin expression correlates with resistance to human skin infections (26). Therefore, this study analyzed the immunomodulatory effects of LL-37 peptides on keratinocytes for comparison with antimicrobial activity. LL-37 had significant effects on the expression of a wide range of transcripts from keratinocytes, with IL-8 showing the most abundant increase in steady
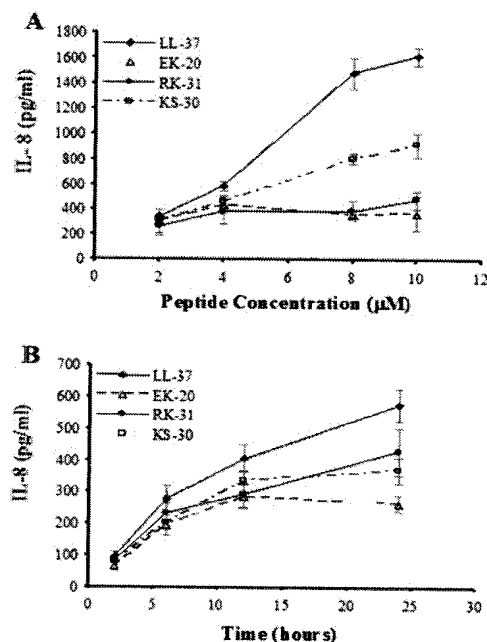
FIGURE 4. Dose response and time course of IL-8 production by human keratinocytes in response to LL-37 and LL-37 peptide fragments. *A*, Peptides were diluted to 2, 4, 8, or 10 μM in keratinocyte medium. Cells were incubated with peptides for 6 h at 37°C. Supernatants were removed and analyzed for IL-8 by ELISA. *B*, Peptides were diluted to 3 μM in keratinocyte medium. Cells were incubated with peptides for 2, 6, 12, or 24 h at 37°C. Supernatants were removed and analyzed for IL-8 by ELISA. Experiments were performed three times in triplicate, with a representative outcome shown as the mean ± SEM.
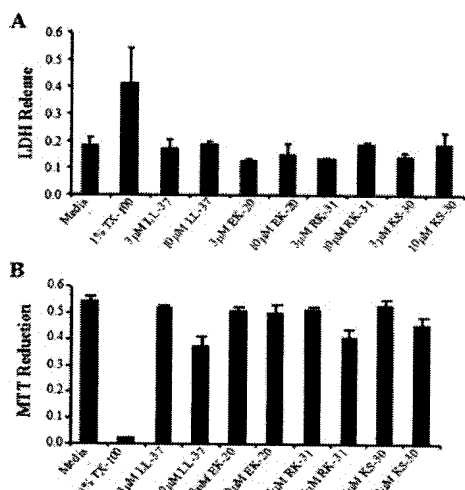
FIGURE 5. Assessment of cytotoxicity to keratinocytes by LL-37 and LL-37 peptide fragments. *A*, Peptides were diluted to 3 or 10 μM in keratinocyte medium. Cells were incubated for 6 h at 37°C, and 50 μl of supernatants were removed and assessed for LDH release. *B*, Peptides were diluted to 3 or 10 μM in keratinocyte medium. Cells were incubated for 6 h at 37°C. Supernatants were removed and replaced with 100 μl of medium. Cells were assessed by MTT assay according to the manufacturer's instructions. Experiments were performed three times in triplicate, with a representative outcome shown as the mean ± SEM.

state transcript abundance by DNA microarray analysis. Several peptides derived from LL-37 and sharing the sequence defined by EK-20 showed potent antimicrobial activity against a wide range
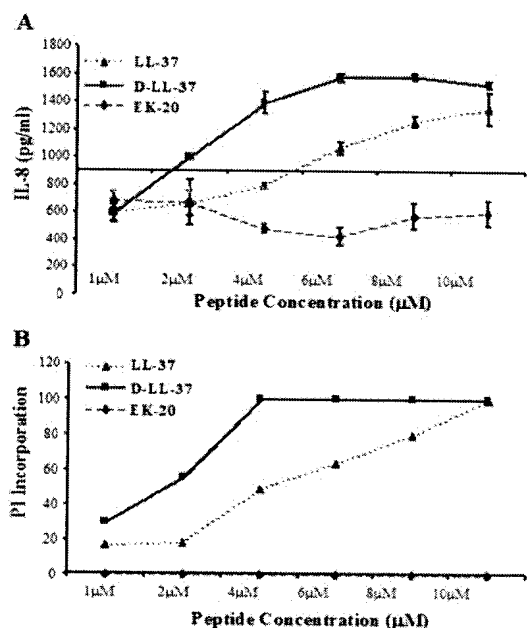

FIGURE 6. Dose response of keratinocyte IL-8 production and membrane permeability in response to LL-37 and D-LL-37 peptides. *A*, Peptides were diluted to 2, 4, 6, 8, or 10 μM in keratinocyte medium. Cells were incubated with peptides for 6 h at 37°C. Supernatants were removed and analyzed for IL-8 by ELISA. *B*, Cells were treated with various peptide doses for 6 h at 37°C. Supernatants were removed and replaced with 1.3 μg/ml PI in PBS. Fluorescence was measured at an excitation of 530 nm and an emission of 620 nm. Experiments were performed three times in triplicate, with a representative outcome shown as the mean ± SEM.
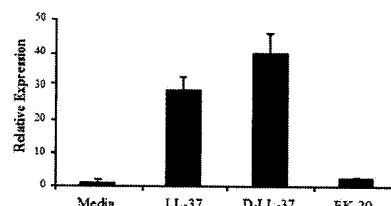

FIGURE 7. Treatment of keratinocytes with LL-37 and D-LL-37 peptides induces IL-8 mRNA expression. Cells were incubated with medium alone, LL-37, D-LL-37, or EK-20 for 6 h at 37C. RNA was extracted using the RNeasy kit, and cDNA was made using the Retroscript kit. Quantitative real-time PCR was performed using TaqMan primers and probes for IL-8 and GAPDH. Experiments were performed three times in triplicate, with a representative outcome shown as the mean ± SEM.

of microorganisms. However, not all peptide fragments of LL-37 that demonstrated antimicrobial activity induced cytokine release by keratinocytes. Furthermore, LL-37 synthesized with amino acids in the D configuration, D-LL-37, was a potent stimulator of IL-8 release by keratinocytes, an effect that was significantly inhibited either by a specific EGFR tyrosine kinase inhibitor, AG1478, or by PTX. Overall, these data suggest that the native processing of cathelicidins to alternate peptide forms can have important consequences for host defense responses.

The findings presented in this study show that LL-37 induces the expression of numerous inflammatory mediators by keratinocytes. The transcripts for IL-8, cyclooxygenase 2, pro-IL-1β, and IL-6 were among those significantly up-regulated. Confirmation of protein expression levels by Luminex assay also showed an increase in the proinflammatory mediators TNF-α and GM-CSF. The Luminex assay did not show an increase in IL-1β synthesis despite the DNA microarray findings and the prior report of IL-1β induction via upstream activation of the P2X7 receptor (20). This finding was not unexpected; although keratinocytes are able to make both IL-1α and IL-1β, they lack the protease required to cleave pro-IL-1β to its active form (27). It is interesting to note that IL-1α is induced after only 1 h of keratinocyte stimulation with LL-37. This immediate response is probably due to the release of stored IL-1α, rather than increased synthesis. The additional increase in IL-1α observed at 24 h probably results from increased synthesis by keratinocytes. The ability of LL-37 to increase proinflammatory mediator release from keratinocytes may have a significant impact on the initial phase of cutaneous inflammation. In fact, epidermal resident cells, including keratinocytes, comprise a major independent component of the acute inflammatory response, which is dictated by chemokine and
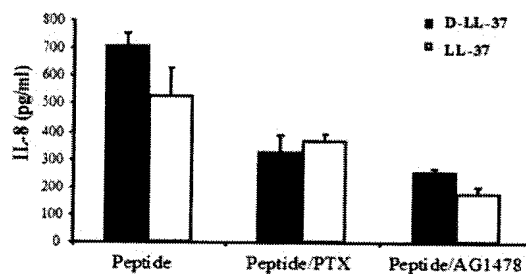

FIGURE 8. PTX and AG1478 inhibit IL-8 induction in response to LL-37 and D-LL-37 peptides. Keratinocytes were pretreated for 1 h with medium alone, 100 ng/ml PTX, or 2 μM AG1478, then stimulated for 6 h at 37°C in the presence or absence of LL-37 or D-LL-37. Supernatants were removed and analyzed for IL-8 by ELISA. Experiments were performed three times in triplicate, with representative outcome shown as the mean ± SEM. Statistical analysis was performed using the paired Student's *t* test.

cytokine expression (28, 29). Before additional cleavage to immunologically inactive forms, early deposition of LL-37 in the epidermis could serve as an additional inflammatory mediator through its properties as a chemoattractant or its ability to stimulate expression of other potent chemotactic agents, such as IL-8.

The results of this study suggest that cathelicidin antimicrobial properties have domain specificity. Specifically, the sequences within EK-20 appeared to be necessary, but not sufficient, for optimal antimicrobial activity. LL-20, LL-15, and LL-12, which lack the sequence VQRIKDFLRN, exhibited the lowest overall antimicrobial activity. The first six or seven amino acids of LL-37 did not appear to be essential for antimicrobial activity, because RK-31 and KS-30 lack these amino acids. Generation of an additional peptide library with site-specific amino acid modifications would serve to better define the domain responsible for cathelicidin antimicrobial activity.

Of principal interest in the current study was the lack of direct correlation between antimicrobial activity and host stimulatory effects, which was indicated by measuring IL-8 production. IL-8 was chosen as the indicator for host immunomodulatory activity due to the high induction of IL-8 mRNA and protein expression in LL-37-treated keratinocytes. In addition, previous investigations have shown that antimicrobial peptides, including LL-37 and neutrophil defensins, induce IL-8 synthesis and release by epithelial cells (21, 30). For example, LL-37, RK-31, and KS-30 exhibited broad-spectrum antimicrobial properties, but stimulated keratinocytes to increase IL-8 production to varying degrees. EK-20, which also exhibited broad-spectrum antimicrobial activity, did not increase IL-8 release. These peptides did not demonstrate significant cytotoxic effects on keratinocytes at concentrations effective for antimicrobial activity or keratinocyte immune stimulation. However, 10 μM LL-37 did affect keratinocyte reduction of MTT, suggesting potential keratinocyte membrane activity at this concentration in culture. Previous structure-function studies of synthetic antimicrobial peptides indicated a dissociation between antimicrobial and immunomodulatory activities (31–33). For example, N-terminal arginine residues of PR-39 were shown to be essential for protein binding and syndecan induction in mammalian cells, whereas modification of C-terminal residues specifically eliminated antimicrobial activity (31). Structural changes to PR-39 influenced antimicrobial activity, whereas mammalian effects were mostly dependent on N-terminal charge. The results of the present study endorse the function of cathelicidin peptides as membrane active agents with relative selectivity for microbial membranes (34). Remarkably, the antimicrobial effects of cathelicidin at concentrations <10 μM extended across bacteria, fungi, and viral envelopes, but spared the eukaryotic plasma membrane of keratinocytes.

The observation that D-LL-37 has more potent immunostimulatory activity than LL-37 raises additional questions pertaining to the mechanism by which peptide treatment induces IL-8 production by keratinocytes. D-LL-37 induced maximal IL-8 release at a low concentration (4 μM), whereas a higher concentration of LL-37 (10 μM) was required for equivalent induction at both the mRNA and protein levels. Interestingly, LL-37 caught up with D-LL-37, reaching the same maximum IL-8 level, albeit at a different dose. The more potent activity of D-LL-37, compared with LL-37, may reflect the resistance to proteolytic degradation of proteins composed of amino acids in the synthetic D form. Thus, additional activity may be a consequence of a higher final concentration after incubation with cells producing active proteases. Furthermore, as expected of membrane-active agents, cell permeability and membrane disruption corresponded directly with IL-8 mRNA and protein production in response to these peptides.

The mechanism by which cathelicidin peptides exert their effects on keratinocytes is unclear. Several receptors have been previously implicated in the host stimulatory effects of LL-37, including FPRL-1, EGFR, and P2X7 (19–21, 23). The current data suggest that specific binding to one of these receptors is an unlikely explanation for the response observed in keratinocytes, because both LL-37 and D-LL-37 induced a maximal IL-8 response. The potent activity of D-LL-37 argues against a highly structure-specific association of cathelicidin peptides with a cell surface receptor, although potential binding to a promiscuous pattern recognition receptor could account for activation by peptides in both the L and D forms, but not by the shorter peptides. Interestingly, treatment with AG1478 and PTX, either independently or in combination, blocked keratinocyte IL-8 induction in response to LL-37 and D-LL-37. Reduced IL-8 production by keratinocytes treated with these inhibitors suggests that the signaling mechanism generated at the membrane by LL-37 and D-LL-37 involves both the EGF receptor and membrane-associated G_i proteins. G_i proteins, which are classically described as being associated with G protein-coupled receptors (GPCRs), are recruited to the intracellular side of the membrane after receptor activation. However, at least three activators of G protein signaling that act independently of these typical receptors have been described (35). Although the data presented in this study suggest that LL-37 peptides permeabilize the cell membrane and are therefore able to enter cells, the ability of these peptides to directly interact with these intracellular activators of G protein signaling was not investigated.

Previous studies have demonstrated ligand-independent transactivation of receptor tyrosine kinases (RTKs), such as EGFR, by GPCRs (36–40). The occurrence of cross-talk between the GPCR and EGFR signaling pathways, which is regulated by the metalloprotease kuzbanian (ADAM10), may help to explain the inhibitory effects of both PTX and AG1478 on the keratinocyte IL-8 response (41, 42). Other studies have suggested that cellular stresses also perturb the cell surface and alter receptor conformation, thereby activating intracellular signaling pathways (36, 37, 43–47). In a similar manner, LL-37 may directly interact with the keratinocyte plasma membrane to cause conformational changes that indirectly activate surface receptor domains linked to intracellular signaling molecules. Although the mechanism by which GPCR and RTK signaling pathways converge has not been completely determined, recent work has suggested that these pathways may run parallel and fulfill different signaling functions (48, 49). Therefore, LL-37-mediated activation of transmembrane receptors, including both GPCRs and RTKs, in addition to influences on keratinocyte membrane ionic equilibrium should be further explored to understand the mechanism by which LL-37 peptides activate downstream production of immunostimulatory molecules by keratinocytes.

These observations support previous studies in several cell, animal, and human systems that cathelicidin peptides have properties of both natural antibiotics and host stimulatory molecules (6, 50–53). The rapid increase in cathelicidin at epithelial interfaces exposed to injury or infection can provide a simultaneous barrier to microbial proliferation and an additional alarm to the host. The term alarmins, which was recently proposed to refer to peptides such as the cathelicidins or defensins, is quite appropriate in this model (54). Proteolytic processing at sites of peptide deposition could enable the epithelia to further modify the spectra of biologic activity of cathelicidin peptides and regulate the balance of activity between host immune modulation and inhibition of microbial growth. Additional studies examining changes in membrane fluidity, ion transport, and receptor aggregation in the presence of LL-37 will offer pertinent information concerning the mechanism by which LL-37 stimulates keratinocytes to produce IL-8 and other proinflammatory molecules.

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